REMARKS

Claims 1-62 are all the claims pending in the application. Claims 25-62 are withdrawn. Applicants herewith amend claims 1, 3, 9, 13, 15, 17 and 21. Support for the Amendment is found at least at paragraphs 42 and 50, 27, 45, 52, 55 and 90, and Examples 3 to 5, respectively of the published specification. Applicants herewith add claims 63-69, support for which is found at least at Examples 3 to 5 and paragraph 45 of the published specification. No new matter is added. Entry of the Amendment is kindly requested.

I. The Title is Proper

At page 2 of the Office Action, the Examiner alleges that the title of the invention is not descriptive. To advance prosecution, Applicants herewith amend the title. The Amendment overcomes the objection.

Withdrawal of the objection is therefore kindly requested.

II. The Claims Are Proper

At page 3 of the Office Action, the Examiner objects to Claims 1-24 because the claims are allegedly not grammatically correct; the claims recite expression of GFP, β galactosidase and cdc-18 but do not recite an operable linkage of the recited genes; the claims recite a reduction in the level of proteolytic degradation and a process which requires that the claimed promoter be expressed in a cell; the claims recite "the said;" the claims are of improper dependent form; and the claims recite functional limitations that are inherently present.

To advance prosecution, the Applicants herewith amend the claims without prejudice or disclaimer. The Amendment overcomes the objections. As to claims 14-24, the objections are moot in light of cancellation of independent claims 14, 16, 18-20 and 22-24.

Withdrawal of the objections is therefore kindly requested.

III. Claims 1 and 7-13 are Patentable Under 35 U.S.C. § 101

At page 5 of the Office Action, the Examiner rejects Claims 1 and 7-13 under 35 U.S.C. § 101 because the claimed invention is allegedly directed to non-statutory subject matter.

To advance prosecution, Applicants herewith cancel or amend the claims without prejudice or disclaimer. The Amendment overcomes the rejection.

Withdrawal of the rejection is therefore kindly requested.

IV. Claims 1-12, 15, 16, and 22 are Definite Under 35 U.S.C. § 112, Second Paragraph

At page 5 of the Office Action, the Examiner rejects Claims 1-12, 15, 16 and 22 under 35 U.S.C. 112, second paragraph, as allegedly being indefinite.

To advance prosecution, the Applicants herewith amend the claims without prejudice or disclaimer. The Amendment overcomes the rejection. Rejection of claims 4, 16, 10 and 22 is moot in light of cancellation of the claims.

Accordingly, withdrawal of the indefiniteness rejection is kindly requested.

V. Claims 2-12 and 14-24 Are Enabled Under 35 U.S.C. § 112, First Paragraph

At page 6 of the Office Action, the Examiner rejects claims 2-12 and 14-24 under 35 U.S.C. § 112, first paragraph. The Office admits that the specification enables temperature regulated promoters selected from the group consisting of SEQ ID NO: 1 and SEQ ID NO: 2, wherein the promoters are operably linked to cdc-18, β-galactosidose, streptokinase or GFP and vectors deposited as MTCC 5106 and 5107. The Office alleges that the specification does not enable any additional embodiment.

Applicants respectfully disagree. In making the rejection, the Examiner first asserts that in the absence of operable linkage of coding sequences for GFP, β-galatosidose, and cdc-18 to promoters, cells containing the promoters are not enabled. Office Action, page 9. To advance prosecution Applicants herewith cancel or amend the claims. Accordingly, Applicants overcome or render moot this aspect of the rejection.

The Examiner further alleges that even if the claims recite distinct structural components not all of the claimed subject matter is enabled because "the specification does not disclose the activity of all of the resulting vectors." Office Action, page 10. The Examiner also states that even though a specific activity is recited, the "ability of the vectors and promoters to reach the specifically recited activity is not shown." Office Action, page 11.

Applicants provide explicit support for multiple combinations of promoters, vectors and genes. For example, the specification sets forth at least 185 and K2 with cdc-18, β-galactosidase, SK and GFP at least at Table 5, Paragraphs 41, 52, 112, 113, 50 and 51; 185 and K3 with GFP and SK at least at paragraph 107 (Figs 4-6), note at paragraph 119, last sentence, and paragraph 56; 185 and K2 with cdc-18 at least at paragraph 108 and 109 and Fig. 7 and 8; 185 and REP3X with β-Galactosidase at least at paragraph 110; 146 and K3 with β-Galactosidase at least at paragraph 53; 146 and K3 with SK at least at paragraph 50 and 51, etc. Furthermore, at paragraph 50 of the published specification pJRK2 and pJRK3 are used with *nmt-145* and *nmt-185*. Applicants' teachings constitute *prima facie* proof enablement.

In addition, the inoperability of a single embodiment does not warrant a finding that the specification fails to enable the claims under 35 U.S.C. § 112, first paragraph. In fact, the Court of Appeals for the Federal Circuit addressed this very issue of enablement when it stated that "[e]ven if some of the claimed combinations were inoperative, the claims are not necessarily invalid. 'It is not a function of the claims to specifically exclude...possible inoperative substances...'" *Atlas Power Co. v. E.I. du Pont de Nemours & Co.*, 750 F.2d 169, 1576 (Fed. Cir. 1984). More to the point, "[t]he presence of inoperative embodiments within the scope of a claim does not necessarily render a claim nonenabled." MPEP, 2164.08(b) 8th Ed. (August, 2005). Thus the Examiner's rejection fails to provide a rational basis necessary to establish a *prima facie* case of non-enablement.

One of ordinary skill in the art would understand that it is common in the art to operably link reporter genes to a promoter in order to provide a quantitative and reliable measure of the strength of a promoter, and that the measure of the strength of the promoter achieved by this method provides an accurate indication as to the level of gene expression expected for numerous genes. Neither the DNA sequence of the open-reading frame, nor the structure of the reporter protein, typically significantly affects promoter activity or nascent polypeptide synthesis.

Applicants herewith attach references which disclose the state of the art as routine and predictive of genetically engineered promoter driven gene expression.[1]

The Examiner lacks legal basis to support the assertion that since the vectors were not shown to express β-galactosidase and since GFP was not demonstrated for pRJK3, that the claims are not enabled. The present facts resemble those under review in *In re Wands*, 858 F.2d 731, (Fed. Cir. 1988), wherein the Court reversed the Examiner's rejection for lack of enablement holding that undue experimentation would not be required to practice the invention because it is known that in producing antibodies it is routine to first make monoclonal hybridomas to determine which hybridomas secrete antibodies with the desired characteristics. The Court found that the specification provided representative working examples as well as the methods needed to practice the invention. Like in the present case, Wands conducted successful experiments and produced evidence that at least one embodiment fell within the scope of the claims. The Court found the claims enabled based on the presence of Applicants' representative examples.

Withdrawal of the lack of enablement rejection is therefore kindly requested.

VIII. Claims 1, 10 and 11 Are Novel Under 35 USC § 102(b)

At page 11 of the Office Action, the Examiner rejects claims 1, 10, 11 under 35 § U.S.C. 102(b) as allegedly being anticipated by Parker et al. (WO 94/03609). The Examiner alleges that Parker et al. teach a promoter having SEQ ID NO:1 and that Parker et al. disclose a reduced level of uninduced protein expression, as compared to CMV or SV40 promoters, as nmt-1 is repressed in the presence of thiamine. Further, the Examiner alleges that the promoter is not deleterious to *S. pombe*.

Applicants respectfully disagree. Applicants' minimal promoters are not taught by Parker et al. In order to establish a *prima facie* case of anticipation the Examiner must prove that

[1] See *Molecular Cloning*, A Laboratory Manual, attached herewith; and *Molecular Biology of the Gene*, Watson et al., attached herewith.

each and every limitation recited by the clam is present in the cited reference. As cited by the Examiner, Parker et al. disclose nmt-1 for expression of a mammalian phospholipids kinase or protein kinase. Parker et al. fails to disclose a minimal promoter regulated by temperature shift from 36°C to 25°C and a maximum expression of genes within three hours of temperature induction. Fig. 6, 9, 10, Examples 2, 3, 5, 6, 7, Tables 2, 3, and 5.

In addition, protein expression using Applicants' nmt-185 and nmt-146 does not impair cellular growth rate - a feature not disclosed by Parker et al. In fact, Parker et al. disclose a reduction in growth rate. Unlike nmt-1, nmt-185 and nmt-146 do not adversely affect host cells.[2]

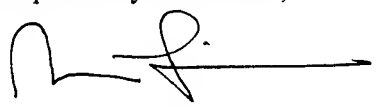
Because all of the elements of Applicants' invention, either implicit or explicit in the claims, are not disclosed by the cited reference, a *prima facie* case of anticipation cannot be set forth. Withdrawal of the rejection is therefore kindly requested.

In view of the above, reconsideration and allowance of this application are now believed to be in order, and such actions are hereby solicited. If any points remain in issue which the Examiner feels may be best resolved through a personal or telephone interview, the Examiner is kindly requested to contact the undersigned at the telephone number listed below.

[2] See, R. Kumar and J. Singh, *Yeast* 23, 55-65 (2006).

The USPTO is directed and authorized to charge all required fees, except for the Issue Fee and the Publication Fee, to Deposit Account No. 19-4880. Please also credit any overpayments to said Deposit Account.

Respectfully submitted,



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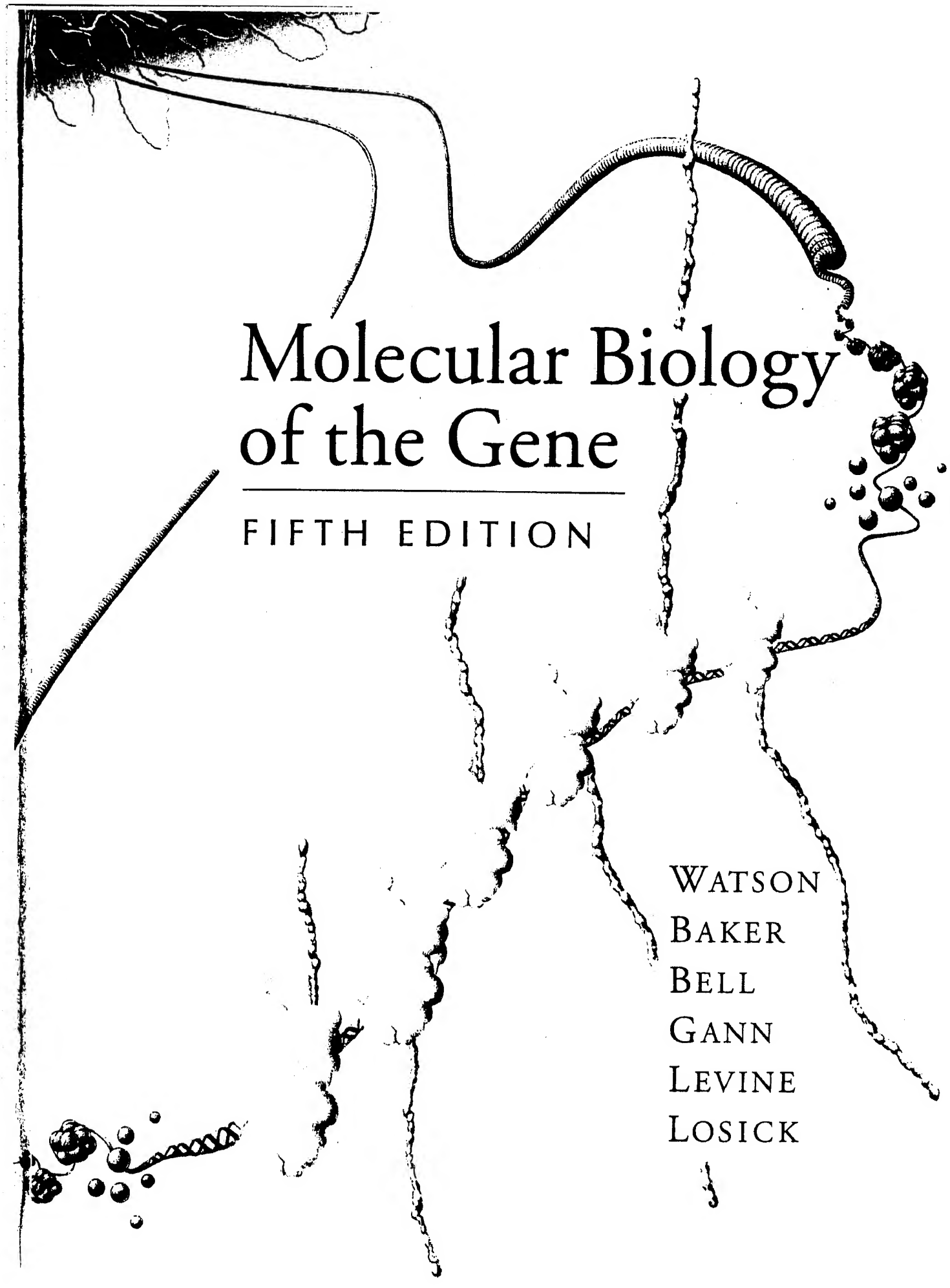

Molecular Biology of the Gene

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tions about the control of gene expression and maintenance of the genome.

DNA Cloning

The ability to construct recombinant DNA molecules and maintain them in cells is called **DNA cloning.** This process typically involves a **vector** that provides the information necessary to propagate the cloned DNA in the cell and an **insert DNA** that is inserted within the vector and includes the DNA of interest. Key to creating recombinant DNA molecules are the restriction enzymes that cut DNA at specific sequences, and other enzymes that join the cut DNAs to one another. By creating recombinant DNA molecules that can be propagated in a host organism, a particular insert DNA can be both purified from other DNAs and amplified to produce large quantities.

In the remainder of this section, we describe how DNA molecules are cut, recombined, and propagated. We then discuss how large collections of such hybrid molecules, called **libraries,** can be created. In a library, a common vector carries many alternative inserts. We describe how libraries are made and how specific DNA segments can be identified and isolated from them.

Cloning DNA in Plasmid Vectors

Once the DNA is cleaved into fragments by a restriction enzyme, it typically needs to be inserted into a vector for propagation. That is, the DNA fragment must be inserted within that second DNA molecule (the vector) to be replicated in a host organism as we described above. By far the most common host used to propagate DNA is the bacterium *E. coli.*

Vector DNAs typically have three characteristics.

1. They contain an origin of replication that allows them to replicate independently of the chromosome of the host.
2. They contain a selectable marker that allows cells that contain the vector (and any attached DNA) to be readily identified.
3. They have single sites for one or more restriction enzymes. This allows DNA fragments to be inserted at a defined point within an otherwise intact vector.

The most common vectors are small (approximately 3 kb) circular DNA molecules that are called **plasmids.** These molecules were originally derived from circular DNA molecules that are found naturally in many bacteria and single-cell eukaryotes (Chapter 21). In many cases, these DNAs carry genes encoding resistance to antibiotics. Thus, naturally occurring plasmids already have two of the characteristics desirable for a vector: they can propagate independently in the host and they carry a selectable marker. A further benefit is that these plasmids are sometimes present in multiple copies per cell. This increases the amount of DNA that can be isolated from a population.

In some cases these plasmids also have useful unique restriction sites. However, since their discovery the plasmids have been simplified and modified such that a typical plasmid vector now has greater than 20 unique restriction sites within a small region. This allows a much more diverse array of restriction enzymes to be used to cut the target DNA. Bacterial viruses—phage—have been modified to allow their use as cloning vectors as well (see Chapter 21).

To insert a fragment of DNA into a vector is a relatively simple process (Figure 20-7). Suppose that a plasmid vector has a unique recognition site for *Eco*RI. Treatment with that restriction enzyme would linearize the plasmid. Because *Eco*RI generates protruding 5′ ends that are complementary to each other (Figure 20-5), the sticky ends are capable of reannealing to re-form a circle with two nicks. Thus, treatment of the circle with the enzyme **DNA ligase** and ATP would seal the nicks to re-form a covalently closed circle.

A target DNA is cleaved with a restriction enzyme to generate potential insert DNAs. Vector DNA that has been cut with the same enzyme is mixed with these insert DNAs and DNA ligase is used to link the compatible ends of the two DNAs. By adding an excess of the insert DNA relative to the plasmid DNA, the majority of vectors will reseal with insert DNA incorporated (Figure 20-7).

Some vectors not only allow the isolation and purification of a particular DNA, but also drive the expression of genes within the insert DNA. These plasmids are called **expression vectors** and have transcriptional promoters immediately adjacent to the site of insertion. If the coding region of a gene (without its promoter) is placed at the site of insertion in the proper orientation, then the inserted gene will be transcribed into mRNA and translated into protein by the host cell. Expression vectors are frequently used to express heterologous or mutant genes to assess their function. They can also be used to produce large amounts of a protein for purification. In addition, the promoter in the expression vector can be chosen such that expression of the insert is regulated by the addition of a simple compound to the growth media (for example, a sugar or an amino acid). This control of when the gene will be expressed is particularly useful if the gene product is toxic.

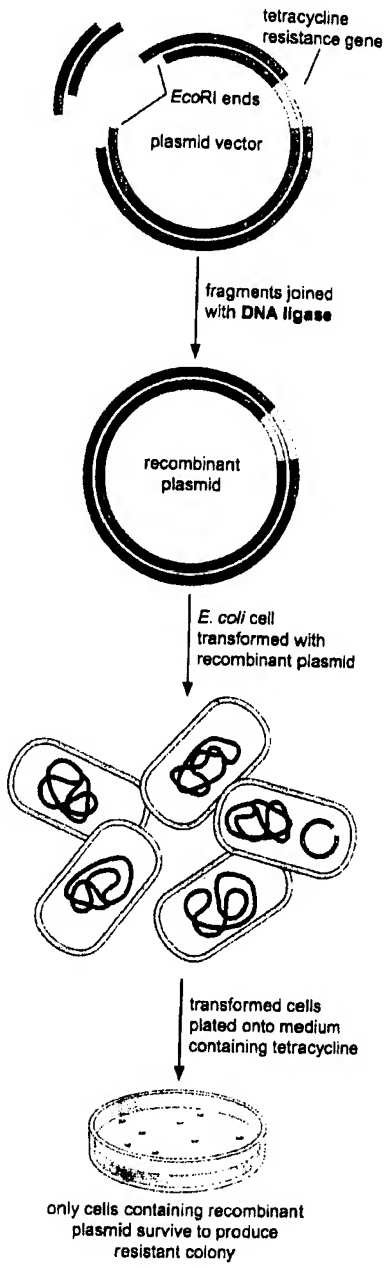

FIGURE 20-7 Cloning in a plasmid vector. A fragment of DNA, generated by cleavage with *Eco*RI, is inserted into the plasmid vector linearized by that same enzyme. Once ligated (see text), the recombinant plasmid is introduced into bacteria, by transformation (see text). Cells containing the plasmid can be selected by growth on the antibiotic to which the plasmid confers resistance. (Source: Adapted from Micklos D.A. and Freyer G.A. 2003. *DNA Science: A first course,* 2nd edition, p. 129, left column. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.)

Vector DNA Can Be Introduced into Host Organisms by Transformation

Propagation of the vector with its insert DNA requires this recombinant molecule be introduced into a host cell by transformation. **Transformation** is the process by which a host organism can take up DNA from its environment. Some bacteria, but not *E. coli*, can do this naturally and are said to have **genetic competence.** *E. coli* can be rendered competent to take up DNA, however, by treatment with calcium ions. Although the exact mechanism for DNA uptake is not known, it is likely that the Ca^{2+} ions shield the negative charge on the DNA, allowing it to pass through the cell membrane. Calcium-treated cells are thus said to be competent to be transformed. An antibiotic to which the plasmid imparts resistance is then used to select transformants that have acquired the plasmid; cells harboring the plasmid will be able to grow in the presence of the antibiotic whereas those lacking it will not.

Transformation generally is a relatively inefficient process. Only a small percentage of the DNA-treated cells take up the plasmid. It is this low efficiency of transformation that makes necessary selection with the antibiotic. After DNA treatment, the cells are transferred onto medium containing the relevant antibiotic and only those cells that have taken up the plasmid and maintain it stably are able to grow.

The inefficiency of transformation also ensures that, in most cases, each cell receives only a single molecule of DNA. This property makes

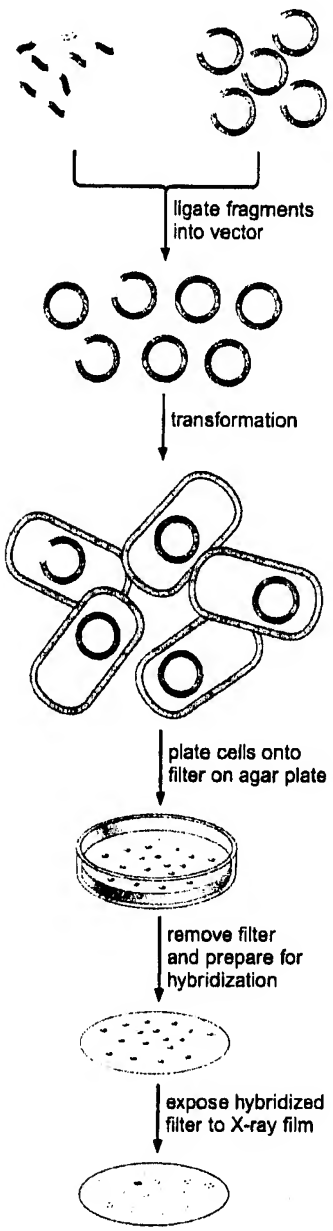

FIGURE 20-8 Construction of a DNA library. To construct the library, genomic DNA and vector DNA, digested with the same restriction enzyme, are incubated together with ligase. The resulting pool or library of hybrid vectors (each vector carrying a different insert of genomic DNA, represented in a different color) is then introduced into *E. coli*, and the cells are plated onto a filter placed over agar medium. Once colonies have grown, the filter is removed from the plate and prepared for hybridization: cells are lysed, the DNA is denatured, and the filer is incubated with a labeled probe. The clone of interest is identified by autoradiography.

each transformed cell and its progeny a carrier of a unique DNA molecule and effectively allows the purification of that molecule away from all other DNAs in the transforming mixture.

Libraries of DNA Molecules Can Be Created by Cloning

It is trivial to generate a specific clone if the starting donor DNA is simple. Thus, if the starting DNA is small (derived from a small virus, for example, with a genome of perhaps only 10 kb), then this can be accomplished simply by separating the DNA fragments after digestion with restriction enzymes and gel electrophoresis. Once separated, DNAs of different sizes can be excised from the gel and purified prior to insertion into a vector.

This is harder to do if the starting DNA is more complex (for example, the human genome). In this case, simple electrophoretic separation of DNA treated with a restriction enzyme will result in very many fragments distributed in a broad range of sizes around the average distance between cut sites. Thus, it is easier under these circumstances to clone the whole population of fragments and separate the individual clones afterwards.

A DNA **library** is a population of identical vectors that each contains a different DNA insert (Figure 20-8). To construct a DNA library, the target DNA (for example, human genomic DNA) is digested with a restriction enzyme that gives a desired average insert size. The insert size can be of any size ranging from less than 100 base pairs to more than a megabase (for very large insert sizes the DNA is typically incompletely cut with a restriction enzyme). The cleaved DNA is then mixed with the appropriate vector cut with the same restriction enzyme in the presence of ligase. This creates a large collection of vectors with different DNA inserts.

Different kinds of libraries are made using insert DNA from different sources. The simplest are derived from total genomic DNA cleaved with a restriction enzyme; these are called **genomic libraries.** This type of library is most useful when generating DNA for sequencing a genome. If, on the other hand, the objective is to clone a DNA fragment encoding a particular gene, a genomic library can be used efficiently only when the organism in question has relatively little noncoding DNA. For an organism with a more complex genome, this type of library is not suitable for this task because many of the DNA inserts will not contain coding DNA sequences.

To enrich for coding sequences in the library, a cDNA library is used. This is made as follows (Figure 20-9). Instead of starting with genomic DNA, mRNA is converted into DNA sequence. The process that allows this is called **reverse transcription** and is performed by a special DNA polymerase (reverse transcriptase) that can make DNA from an RNA template (see Chapter 11). When treated with reverse transcriptase, mRNA sequences can be converted into double-stranded DNA copies that are called **cDNAs** (for **copy DNAs**). These fragments are then ligated into the vector.

To isolate individual inserts from a library, *E. coli* cells are transformed with the entire library. Each transformed cell typically contains only a single vector with its associated insert DNA. Thus, each cell that propagates after transformation will contain multiple copies of just one of the possible clones from the library. The colony produced from cells carrying any cloned sequence of interest can be

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Molecular Cloning

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BECAUSE OF THE VAST FUND OF KNOWLEDGE ABOUT ITS GENETICS, biochemistry, and molecular biology, *Escherichia coli* is the system of first choice for expression of many heterologous proteins: Genetic manipulations are straightfoward, cultures of *E. coli* are easily and inexpensively grown, and many foreign proteins are well-tolerated and may be expressed at high levels. During the past 20 years, several hundred recombinant proteins have been expressed in *E. coli* using one or another of the vector systems described in this chapter. However, *E. coli* is not always the host of choice. For example, proteins whose full biological activity requires posttranslational modification (e.g., glycosylation or cleavage at specific sites) may best be expressed in a eukaryotic host. It is therefore important before embarking on an expression project to assess the final goal and to determine which host-vector system may be most appropriate. These systems are reviewed in the information panel on EXPRESSION OF CLONED GENES at the end of this chapter.

CHOOSING AN EXPRESSION SYSTEM

Factors that influence the choice of a system for expression of a particular protein in *E. coli* are listed below.

- ***The size of the protein.*** Small cytosolic proteins and polypeptides (<100 residues in length) are best expressed in *E. coli* as fusion proteins composed of carrier sequences linked by a standard peptide bond to the target protein (please see section on Fusion Proteins, p. 15.4). The carrier often stabilizes the protein of interest against intracellular degradation and provides a ligand-binding site that can be used for affinity purification. The target protein may frequently be recovered in an active form by including a proteolytic cleavage site at an appropriate location in the fusion protein.

  Cytosolic proteins >100 residues in length are the most problematic proteins to express in either system. In *E. coli*, these proteins are often unstable or form insoluble inclusion bodies. In mammalian cells, problems can arise in distinguishing the foreign protein from its endogenous homolog.

- ***The amount of protein needed.*** If only small quantities of the target protein are required — for example, when screening a series of site-directed mutants for enzymatic activity — there is little point in trying to optimize production. Most of the standard expression plasmids can be used successfully if the enzyme can be assayed in crude extracts of *E. coli*. However, if purification of an active protein is necessary and/or if the protein is required in large quantities, it is usually necessary to explore several different host-vector systems and purification schemes before finding one that is workable on a large scale.

- ***Whether active protein is required.*** If the purpose of expressing the target protein is simply to obtain material for raising antibodies, there is no point in trying to obtain active protein. Instead, expression systems can be used that facilitate purification of the target protein, irrespective of its state of biological activity or denaturation. Here, the formation of inclusion bodies is a great advantage for isolating insoluble protein, as are tags that can be used during affinity purification of the target protein.

  If the target protein is to be used in biochemical or cell biological studies, then maintaining or restoring protein function is important and ease of purification matters less. In some cases, direct expression vectors may be used to produce soluble, active proteins. In most cases, however, the expressed protein will be insoluble and must be purified from inclusion bodies, solubilized, and refolded into an active form.

When the expressed protein is to be used in structural studies, it is best to express the target as a soluble protein. It may be necessary to test expression in several different strains of *E. coli* to establish conditions that minimize misfolding in vivo, and to take great care to minimize denaturation in vitro to maintain the proper configuration of the protein (please see the panel on ADDITIONAL PROTOCOL: REFOLDING SOLUBILIZED PROTEINS RECOVERED FROM INCLUSION BODIES in Protocol 8).

CHOOSING A PROMOTER AND VECTOR SYSTEM

The following categories of expression vectors, based on the type of promoter, are described in detail in the first series of protocols in this chapter (please also see the information panel on *E. COLI* EXPRESSION SYSTEMS).

Expression Vectors Containing an IPTG-inducible Promoter (Protocol 1)

Several different vectors based on the *lac* operon are used for high-level expression of foreign proteins in *E. coli*, including:

- ***The* trp-lac *(tac) promoter.*** *tac* is a hybrid *trp-lac* promoter containing the –35 region of the *trp* promoter fused to the –10 region of the *lac*UV5 promoter; it is regulated by the *lac* repressor and is independent of cAMP regulation mediated by the *crp* gene product (Amann et al. 1983; de Boer et al. 1983). A useful *tac* promoter expression plasmid (pKK223-3 [Brosius and Holy 1984]) is available from Pharmacia.

- ***The* trp-lac *(trc) promoter.*** *trc* is another version of the *lac* repressor-regulated hybrid *trp-lac* promoter containing the –35 region of the *trp* promoter fused to the –10 region of the *lac*UV5 promoter (Amann and Brosius 1985). The only difference between the *trc* and *tac* promoters is the distance separating the –35 and –10 regions of the promoter. In the *trc* promoter, these two elements are separated by a consensus distance (17 bp), whereas in the *tac* promoter, they are separated by 16 bp. This difference has little or no effect on the expression levels of foreign proteins (Amann and Brosius 1985). The expression plasmid p*Trc* 99A, available from Pharmacia, carries the *trc* promoter.

- ***The* lac *promoter.*** Any general-purpose vector (pUC, pTZ, pSK, pBluescript, pGEM, etc.) designed for blue/white screening for clones containing inserts of foreign DNA can be used to express a foreign protein, usually as a fusion protein with amino acids encoded by the amino terminus of the *lacZ* gene and/or the polylinker sequence (for further details, please see the information panel on LACZ FUSIONS). Although the *lac* promoter is not as strong as the *tac* or *trc* promoters, the high copy number of most general-purpose vectors allows expression of foreign proteins at respectable levels. Maximum induction of the *lac* promoter requires the action of the cAMP activator protein (CAP, the *crp* gene product), which is most active when cells are grown in medium lacking glucose. Media that contain glucose as a carbon source should not be used to express genes cloned into these vectors.

Expression Vectors Containing the Bacteriophage T7 Promoter (Protocol 2)

The pET series of vectors, originally developed by Studier et al. (1990) and since expanded, allow regulated expression of foreign genes by bacteriophage T7 RNA polymerase. These vectors typically carry the colicin E1 (colE1) replicon of pBR322 and confer resistance to ampicillin or kanamycin. Their multiple cloning sites allow an inserted coding sequence to be placed under

control of the "natural" promoter for T7 RNA polymerase (the ϕ10 promoter), or under the control of the so-called "T7*lac*" promoter, a derivative of the natural promoter that has the *lac* operator (*lacO*) placed so that binding of the *lac* repressor blocks transcription initiation.

Expression Vectors Containing the Bacteriophage λ p_L Promoter (Protocol 3)

In vectors of this class, the bacteriophage λ p_L promoter is regulated by a temperature-sensitive repressor, *cIts*857, which represses p_L-driven transcription at low temperatures but not at elevated temperatures. p_L vectors are particularly useful if the expressed gene product is toxic to *E. coli*. Several p_L vectors are commercially available including the pHUB series (Bernard and Helinski 1979), pPLc series (Remaut et al. 1981), pKC30 (Rao 1984), pAS1 (Rosenberg et al. 1983), pRM1/pRM9 (Mieschendahl et al. 1986), and pTrxFus (LaVallie et al. 1993). *E. coli* strains (e.g., M5219) harboring the *cIts*857 mutation must be used as hosts for expression vectors carrying the bacteriophage λ p_L promoter.

FUSION PROTEINS

Gene fusions are created by joining together two or more open reading frames in a desired order. Expression of fused reading frames generates hybrid proteins in which the protein of interest is attached to the amino terminus or the carboxyl terminus of a carrier protein (Itakura et al. 1977; Goeddel et al. 1979; for review, please see Uhlén and Moks 1990; LaVallie and McCoy 1995). Fusion proteins have a vast array of potential uses.

- ***Attaching target proteins to a domain*** of known enzymatic function and/or antigenic composition may provide a convenient method to "tag" and isolate the target protein sequences.
- ***Joining the target protein to topogenic signals*** may allow the fusion protein to be directed to specific cellular compartments.
- ***Adding "carrier" sequences*** may protect the target protein from proteolysis in prokaryotic hosts.
- ***Adding carrier sequences*** may improve the solubility of the target protein and may prevent the formation of insoluble inclusion bodies (please see the following section on Dealing with Insoluble Proteins).

Table 15-1 contains a summary of vectors widely used for creation and expression of fusion proteins.

Purification of Fusion Proteins

By fusing the polypeptide of interest to a carrier that has high affinity for a specific ligand, almost any fusion protein can be purified by affinity chromatography, often in a single step. More than 20 different fusion systems have been designed for, or adapted to, affinity purification. The primary features of a vector used for constructing a fusion protein are shown in Figure 15-1. Many of these fusion vectors are based on LacZ, but other carrier proteins that have been used successfully include *malE*, glutathione-*S*-transferase, and staphylococcal protein A. Recently, identification tags derived from well-characterized ligand-binding proteins have been partially supplanted by artificial tags that have no counterpart in the natural world. The best known of these are (1) a polyhistidine sequence that binds to columns carrying Zn^{2+} or Ni^{2+} (Smith et al. 1988) and (2) FLAG, a designer heptapeptide that is both hydrophilic and immunoreactive. The five carboxy-

TABLE 15-1 Vectors for Construction of Fusion Proteins

VECTOR SYSTEM	FUSION PARTNER[a]	COMMENTS	REFERENCE/SOURCE
pUC, pSK, pBluescript, pGEM	β-galactosidase	Expression under control of the *lac* promoter-operator system (please see the information panel on LACZ FUSIONS).	Stratagene (www.stratagene.com) Life Technologies (www.lifetech.com)
pTA1529 or pBAce	alkaline phosphatase	PhoA signal sequence facilitates transport to periplasm.	Oka et al. (1985)
pGEX series	GST	IPTG-inducible promoter; available with cleavage sequences.	Smith and Johnson (1988) Pharmacia
pMAL series	MBP	IPTG-inducible promoter; MBP signal sequence facilitates export to periplasm.	di Guan et al. (1988); Maina et al. (1988) New England Biolabs (www.neb.com)
pTrx, pTrxFus	Trx	IPTG-inducible promoter; available with enterokinase cleavage sequence.	LaVallie et al. (1993) Invitrogen (www.invitrogen.com)
pET series	poly-His tag, selected vectors also carry tags for GST, Trx, DsbA, and DsbC, CBD	T7 promoter (IPTG-inducible); avail- with sites for chemical, enzymatic cleavage.	Studier et al. (1990) Novagen (www.novagen.com) Promega (www.promega.com)

[a](GST) Glutathione *S*-transferase; (MBP) maltose-binding protein; (Trx) thioredoxin; (DsbA and DsbC) disulfide bond formation (periplasmic localization); (CBD) cellulose-binding domain.

terminal amino acids of FLAG constitute a recognition site for the protease enterokinase, which can be used to remove the peptide from the target protein (Dykes et al. 1988; Hopp et al. 1988). Table 15-2 lists a number of fusion systems, each of which has been used successfully in several laboratories for affinity purification of different fusion proteins.

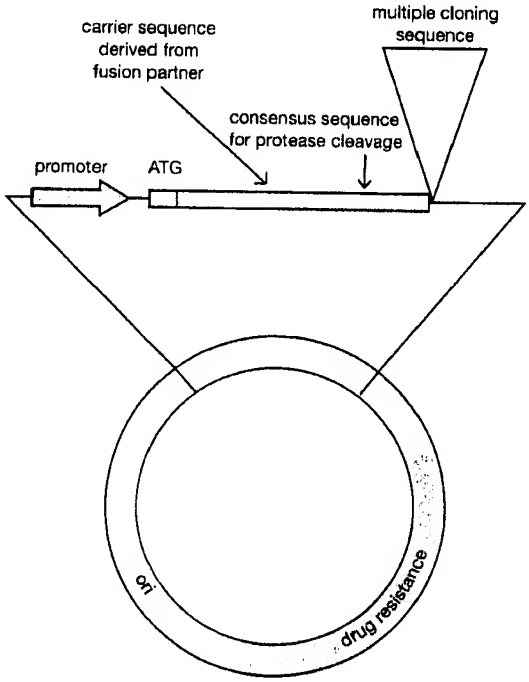

FIGURE 15-1 Schematic of a Generic Fusion Vector

TABLE 15-2 Affinity Purification of Fusion Proteins

Carrier Protein	Affinity Ligand[a]	Method of Elution	References
β-galactosidase	APTG TPEG	sodium borate (pH 10)	Germino et al. (1983); Ullmann (1984)
Protein A	IgG	0.5 M acetic acid	Nilsson et al. (1985a); Moks et al. (1987a,b); Nilsson and Abrahmsén (1990)
Glutathione-*S*-transferase	glutathione	reduced glutathione (5 mM)	Smith and Johnson (1988)
Maltose-binding protein	cross-linked amylose	maltose (10 mM)	di Guan et al. (1988); Maina et al. (1988)
Chloramphenicol acetyltransferase	*p*-aminochloram-phenicol-Sepharose	chloramphenicol (5 mM)	Knott et al. (1988)
Carbonic anhydrase II	sulfonamide affinity resin	Tris-SO_4 (pH 6.8)	Van Heeke et al. (1993)
Cellulose-binding domain	cellulose	H_2O	Ong et al. (1989a,b)
Poly(histidine)	immobilized Zn^{2+} or Ni^{2+}	acid gradient (pH 6.0 to pH 4.0) imidazole (up to 0.5 M)	Smith et al. (1988)
FLAG	antibody specific for FLAG	EDTA at neutral pH or glycine buffer at pH 3.0	Hopp et al. (1988)
Poly(arginine)	S-Sepharose	gradient of NaCl	Brewer and Sassenfeld (1985)
Poly(cysteine)	thiopropyl-Sepharose	mercaptoethanol or dithiothreitol	Persson et al. (1988)
Poly(phenylalanine)	phenyl-Superose	ethylene glycol	Persson et al. (1988)

[a]TPEG and APTG are acronyms for the same compound: *p*-aminophenyl-β-D-thio-galactoside, which was first used for purification of β-galactosidase by Steers et al. (1971).

Fusion proteins produced in *E. coli* are often excellent immunogens that can be used to raise antisera against the target sequences. However, in many cases, the penalty for attaching a ligand-binding domain to the target sequences is loss of biological activity.

Cleavage of Fusion Proteins

To obtain the polypeptide of interest in a native and biologically active form, it must be cleaved from the remainder of the fusion protein. Although both chemical and enzymatic methods have been developed to cleave peptide bonds at the joint between the sequences of the target protein and the carrier protein, efficient removal of the tag or carrier protein remains a major problem. Chemical methods are specific to a particular amino acid or small group of amino acids. For example, cyanogen bromide cleaves only at methionine residues, whereas formic acid cleaves only at proline residues that are preceded by aspartic acid residues (please see Table 15-3). If these potential sites are present in the joint region of the fusion protein and are absent from the target sequences, chemical cleavage can be used to release intact target sequences from fusion proteins.

Cyanogen bromide, for example, has been used in the production of somatostatin (Itakura et al. 1977) and in the processing of β-galactosidase-insulin A chain fusion and β-galactosidase-insulin B chain fusion (Goeddel et al. 1979). In addition, the polylinker sequences of many expression plasmids contain a *Bam*HI site, 5′-NGGATCCN-3′. If this sequence of two codons (GAT and CCN), which encode the dipeptide AspPro, is in-frame with the fusion protein, then formic acid can be used to cleave the polypeptide (Table 15-3). In practice, however, chemical

cleavage is of limited use since most target proteins contain one or more potential cleavage sites. In addition, chemical cleavage is rarely as specific as it should be and is generally carried out under harsh reaction conditions that tend to denature the target protein. In most cases, therefore, the target protein is obtained in poor yield and in an inactive state.

Target proteins can sometimes be separated from the carrier sequences by enzymatic cleavage. This requires planning because the fusion protein must be designed so that it contains a specific proteolytic cleavage site at the joint between the target sequences and the carrier protein. This problem has been greatly ameliorated by the development of commercially available vectors containing a polycloning site downstream from the sequences coding for the carrier protein and a proteolytic cleavage site. For example, in the pMAL2 family of vectors, the coding sequence of interest is fused in-frame to the 3′ end of a synthetic sequence encoding the four-amino-acid recognition site for Factor Xa (Ile-Asp/Glu-Gly-Arg) (Nagai and Thøgerson 1984; Nambiar et al. 1987). Upstream of this sequence is a segment of DNA encoding the *E. coli* MalE protein. Factor Xa cleaves the peptide bond that is carboxy-terminal to the arginine residue in the recognition site (see Figure 15-2).

By carefully choosing the restriction sites that will be used to insert the target sequences into the vector, it is possible to arrange that few, if any, foreign amino acids remain attached to the amino terminus of the target protein after cleavage. Similar strategies can be used with many of the other enzymatic cleavage systems listed in Table 15-3.

Although this method of purifying the sequences of the protein of interest from the fusion protein looks great on paper, it works cleanly only ~50% of the time. With many enzymes, the efficiency of the proteolytic cleavage reaction is poor unless the fusion protein has been denatured with 6 M guanidine hydrochloride or 8 M urea or after a "spacer" has been inserted on one or both sides of the cleavage site. In other cases, because the specificity of the protease is not absolute or because the enzyme used for cleavage is contaminated by other proteases, the protein of interest is cleaved internally at sites that are related to the recognition site (Nagai and Thøgerson 1984, 1987; Dykes et al. 1988; Lauritzen et al. 1991). Problems of this type can sometimes, but not always, be solved by changing proteases or by using vectors (e.g., those coding for glutathione reductase or thioredoxin fusions) that typically express fusion proteins in a soluble form rather than in an inclusion body.

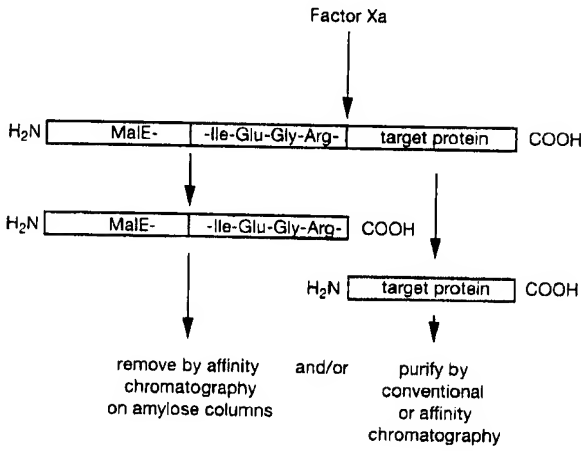

FIGURE 15-2 Cleavage of Fusion Proteins by Factor Xa

Factor Xa cleaves the peptide bond that is carboxy-terminal to the arginine residue in the recognition site.

Because few direct comparisons of the efficiency of cleavage of specific proteins by different proteases have been published, it is impossible to recommend one system over another with confidence. However, in one comparative study, the TEV (tobacco etch virus) protease was far more efficient at cleaving glutathione-*S*-transferase- and maltose-binding protein fusions than Factor Xa. TEV protease also has advantages over other proteases: Cleavage at cryptic sites occurs rarely if at all; the enzyme is fully active in the presence of common inhibitors of proteases and works at low temperatures (4–30°C) (Polayes et al. 1994). Finally, the TEV protease is itself available (from Life Technologies) as a fusion protein carrying a polyhistidine tag. This simplifies removal of the protease after cleavage of the fusion protein. The chemical and enzymatic reactions that have been used to achieve specific cleavage of peptide bonds in fusion proteins are shown in Table 15-3.

TABLE 15-3 Cleavage of Fusion Proteins

Method of Cleavage	Sequence at Cleavage Site	Name of Vector(s)	References
Chemical			
Cyanogen bromide and 70% formic acid	-Met↓		Itakura et al. (1977); Szoka et al. (1986)
Formic acid (70%) and heat	-Asp↓Pro-		Nilsson et al. (1985b); Szoka et al. (1986); Boutelje et al. (1990)
Hydroxylamine at pH 9 and heat	-Asn↓Gly-		Moks et al. (1987b); Forsberg et al. (1990); Canova-Davis et al. (1992); Edalji et al. (1992); King et al. (1992)
Iodosobenzoic acid 2-(2-nitrophenyl)-3-methyl-3-bromoindolenine in 50% acetic acid (BNPS-skatole)	-Trp↓		Dykes et al. (1988); Knott et al. (1988); Villa et al. (1988)
Enzymatic			
Ala-64 subtilisin[a]	-Gly-Ala-His-Arg↓		Forsberg et al. (1991, 1992)
Clostripain	-Arg↓ and Lys-Arg↓		Bennett et al. (1984)
Collagenase	-Pro-Val↓Gly-Pro-		Germino and Bastia (1984); Lee and Ullrich (1984); Hiraoka et al. (1991); Chinery et al. (1993)
Enterokinase	-Asp-Asp-Asp-Asp-Lys↓		Dykes et al. (1988); Hopp et al. (1988); Su et al. (1992); Van Heeke et al. (1993)
Factor Xa	-Ile-Glu (or Asp)-Gly-Arg↓	pMal-c2 (cytosolic) pMal-p2 (periplasmic) pGEX3X (Pharmacia)	Nagai and Thøgerson (1984, 1987); Lauritzen et al. (1991); Ohashi et al. (1991)
Renin	-Pro-Phe-His-Leu↓Leu-		Haffey et al. (1987)
α-Thrombin[b]	-Leu-Val-Pro-Arg↓Gly-Ser-	pGEX2T (Pharmacia)	Gearing et al. (1989)
Trypsin	-Arg↓ or -Lys↓		Shine et al. (1980); O'Hare et al. (1990); Wang et al. (1989)
TEV protease[c] (tobacco etch virus protease)	-Glu-Asn-Leu-Tyr-Phe-Gln↓Gly-	pPROEX-1 (Life Technologies)	Parks et al. (1994); Polayes et al. (1994)

[a]Ala-64 subtilisin is a site-directed mutant of subtilisin in which the catalytic His-64 is replaced by alanine (H64A). The mutant enzyme is very specific for substrates containing a histidine (Carter and Wells 1987).

[b]The optimum cleavage sites for α-thrombin are (1) P4-P3-Pro-Arg-P1′-P2′, where P3 and P4 are hydrophobic amino acids and P1′ and P2′ are nonacidic amino acids, and (2) P2-Arg-P1′, where P2 or P1′ is Gly (Chang 1985).

[c]The Glu, Tyr, Gln, and Gly residues are required for cleavage (Carrington and Dougherty 1988; Dougherty et al. 1988; Dougherty and Parks 1989).

Chapter 16

Introducing Cloned Genes into Cultured Mammalian Cells

A VARIETY OF STRATEGIES ARE AVAILABLE FOR THE DELIVERY of genes into eukaryotic cells. These techniques fall into three categories: transfection by biochemical methods, transfection by physical methods, and virus-mediated transduction. This chapter deals with the first two categories; the third approach is covered extensively in Section 8 of *Cells: A Laboratory Manual* (Spector et al. 1998b). The choice of a particular transfection method is determined by the experimental goal (e.g., the type of assay to be used for screening, the ability of the cell line to survive the stress of transfection, and the efficiency required of the system).

Biochemical methods of transfection, including calcium-phosphate-mediated and diethylaminoethyl (DEAE)-dextran-mediated transfection, have been used for nearly 30 years to deliver nucleic acids into cultured cells. The work of Graham and van der Eb (1973) on transformation of mammalian cells by viral DNAs in the presence of calcium phosphate laid the foundation for the biochemical transformation of genetically marked mouse cells by cloned DNAs (Maitland and McDougall 1977; Wigler et al. 1977), for the transient expression of cloned genes in a variety of mammalian cells (e.g., please see Gorman et al. 1983b), and for the isolation and identification of cellular oncogenes, tumor-suppressing genes, and other single-copy mammalian genes (e.g., please see Wigler et al. 1978; Perucho and Wigler 1981; Weinberg 1985; Friend et al. 1988). More recently, a collection of cationic lipid (liposome) reagents has been used successfully for gene delivery into a wider range of cell types. In all three of these chemical methods (calcium phosphate, DEAE-dextran, and cationic lipids), the chemical agent forms a complex with the DNA that facilitates its uptake into cells.

Two physical methods of transfection are in common use: Biolistic particle delivery and direct microinjection work by perforation of the cell membrane and subsequent delivery of the DNA into the cell; electroporation uses brief electrical pulses to create transient pores in the plasmid membrane through which nucleic acids enter.

TRANSIENT VS. STABLE TRANSFECTION

Two different approaches are used to transfer DNA into eukaryotic cells: transient transfection and stable transfection. In transient transfection, recombinant DNA is introduced into a recipient cell line in order to obtain a temporary but high level of expression of the target gene. The transfected DNA does not necessarily become integrated into the host chromosome. Transient transfection is the method of choice when a large number of samples are to be analyzed within a short period of time. Typically, the cells are harvested between 1 and 4 days after transfection, and the resulting lysates are assayed for expression of the target gene.

Stable or permanent transfection is used to establish clonal cell lines in which the transfected target gene is integrated into chromosomal DNA, from where it directs the synthesis of moderate amounts of the target protein. In general (depending on the cell types), the formation of stably transfected cells occurs with an efficiency that is one to two orders of magnitude lower than the efficiency of transient transfection. Isolation of the rare stable transformant from a background of nontransfected cells is facilitated by the use of a selectable genetic marker. The marker may be present on the recombinant plasmid carrying the target gene, or it may be carried on a separate vector and introduced with the recombinant plasmid into the desired cell line by cotransfection (for further details, please see the information panels on **COTRANSFORMATION** and **SELECTIVE AGENTS FOR STABLE TRANSFORMATION**). In general, all of the methods described below are suitable for use in transient transfection assays, and all, with the exception of DEAE-dextran, may be used for stable transfection.

TRANSFECTION METHODS

Until recently, cloned DNA has been introduced into cultured eukaryotic cells chiefly by biochemical methods. During the past 10 years, the range of cell types that can be transfected efficiently has been extended with the development of liposome methods, which work well with suspension cultures, and with the use of physical methods such as electroporation and biolistic particle delivery, which may be used successfully with many cell lines that are resistant to transfection by other means. A brief summary of transfection methods is given in Table 16-1.

TABLE 16-1 Transfection Methods

Method	Expression		Cell Toxicity	Cell Types	Comments
	Transient	Stable			
Lipid-mediated **Protocol 1**	yes	yes	varies	adherent cells, primary cell lines, suspension cultures	Cationic lipids are used to create artificial membrane vesicles (liposomes) that bind DNA molecules. The resulting stable cationic complexes adhere to and fuse with the negatively charged cell membrane (Felgner et al. 1987; Felgner et al. 1994).
Calcium-phosphate-mediated **Protocols 2 and 3**	yes	yes	no	adherent cells (CHO, 293); suspension cultures	Calcium phosphate forms an insoluble coprecipitate with DNA, which attaches to the cell surface and is absorbed by endocytosis (Graham and van der Eb 1973).
DEAE-dextran-mediated **Protocol 4**	yes	no	yes	BSC-1, CV-1, and COS	Positively charged DEAE-dextran binds to negatively charged phosphate groups of DNA, forming aggregates that bind to the negatively charged plasma membrane. Uptake into the cell is believed to be mediated by endocytosis, which is potentiated by osmotic shock (Vaheri and Pagano 1965).
Electroporation **Protocol 5**	yes	yes	no	many	Application of brief high-voltage electric pulses to a variety of mammalian and plant cells leads to the formation of nanometer-sized pores in the plasma membrane (Neumann et al. 1982; Zimmermann 1982). DNA is taken directly into the cell cytoplasm either through these pores or as a consequence of the redistribution of membrane components that accompanies the closure of the pores. Electroporation can be extremely efficient and may be used for both transient and stable transfection.
Biolistics **Protocol 6**	yes	yes	no	primary cell lines; tissues, organs, plant cells	Small particles of tungsten or gold are used to bind DNA, in preparation for delivery into cells, tissues, or organelles by a particle accelerator system (Sanford et al. 1993). This process has been variously called the microprojectile bombardment method, the gene gun method, and the particle acceleration method. Biolistics is used chiefly to transform cell types that are impossible or very difficult to transform by other methods.
Polybrene **Protocol 7**	yes	yes	varies	CHO and keratinocyte	The polycation Polybrene allows the efficient and stable introduction of low-molecular-weight DNAs (e.g., plasmid DNAs) into cell lines that are relatively resistant to transfection by other methods (Kawai and Nishizawa 1984; Chaney et al. 1986; Aubin et al. 1997). The uptake of DNA is enhanced by osmotic shock and dimethylsulfoxide (DMSO), which may permeabilize the cell membrane.

TRANSFECTION CONTROLS

All transfection experiments should include controls to test individual reagents and plasmid DNA preparations, and to test for toxicity of the gene or construct being introduced.

Controls for Transient Expression

Negative Controls

In transient transfection experiments, one or two dishes of cells should be transfected with the carrier DNA and/or buffer used to dilute the test plasmid or gene. Typically, salmon sperm DNA or another inert carrier such as the vector used to construct the recombinant is transfected into adherent cells in the absence of the test gene. After transfection, the cultured cells should not detach from the dish nor become rounded and glassy in appearance.

Positive Controls

One or two dishes of cells are transfected with a plasmid encoding a readily assayed gene product such as chloramphenicol acetyl transferase, luciferase, *Escherichia coli* β-galactosidase, or green fluorescent protein, whose expression is driven by a pan-specific promoter such as the human cytomegalovirus immediate early gene region promoter and enhancer. Tracer plasmids of this kind are available from many different commercial suppliers who sell kits containing the enzymes and reagents needed for detection of the encoded protein. Because the endogenous levels of these reporter activities are typically low, the increase in enzyme activity provides a direct indication of the efficiency of the transfection and the quality of the reagents used for a particular experiment. This control is especially important when comparing results of transfection experiments carried out at different times. Cotransfecting the reporter plasmid with the test plasmid or genomic DNA also provides a control for nonspecific toxicity in the overall transfection process.

Controls for Stable Expression

Negative Controls

One or two dishes should be transfected with an inert nucleic acid such as salmon sperm DNA, in the absence of the selectable marker. After culturing for 2–3 weeks in the presence of the selected agent (G418, hygromycin, mycophenolic acid), no colonies should be visible.

Positive Controls

One or two dishes of cells should be transfected with the plasmid encoding the selectable marker in the absence of any other DNA. The number of viable colonies present at the end of the 2–3-week selection period is a measure of the efficiency of the transfection/selection process. A similar number of colonies should be present on dishes into which both the selectable marker and the test plasmid or gene were introduced. A marked discrepancy in the number of colonies on these two sets of dishes can be an indication of a toxic gene product (or in rare instances of a gene product that enhances survival of the transfected cells). If a particular cDNA or gene proves toxic to

recipient cells, consider the use of a regulated promoter such as metallothionein (a Zn^{2+}- or Cd^{2+}-responsive DNA), the mouse mammary tumor virus long terminal repeat promoter (a glucocorticoid-responsive DNA), a tetracycline-regulated promoter (Gossen and Bujard 1992; Gossen et al. 1995; Shockett et al. 1995), or an ecdysone-regulated system (No et al. 1996). Alternatively, conditional alleles of some genes can be constructed (Picard et al. 1988).

OPTIMIZATION AND SPECIAL CONSIDERATIONS

Irrespective of the method used to introduce DNA into cells, the efficiency of transient or stable transfection is determined largely by the cell type that is used (please see Table 16-1). Different lines of cultured cells vary by several orders of magnitude in their ability to take up and express exogenously added DNA. Furthermore, a method that works well for one type of cultured cell

TABLE 16-2 Commercial Kits and Reagents for Transfection

MANUFACTURER	WEBSITE ADDRESS	KIT/PRODUCT	METHOD OR REAGENTS
Amersham-Pharmacia Biotech	www.apbiotech.com	CellPhect Transfection Kit	$CaPO_4$ or DEAE-Dextran
Bio-Rad	www.biorad.com	CytoFectene Reagent	Cationic lipid
CLONTECH	www.clontech.com	CLONfectin Reagent	Cationic lipid
		CalPhos Mammalian Kit	$CaPO_4$
5 Prime→3 Prime	www.5prime.com	Calcium Phosphate Transfection Kit	$CaPO_4$ DEAE-Dextran
Invitrogen	www.invitrogen.com	Perfect Lipid	Cationic lipid
Life Technologies	www.lifetech.com	Lipofectamine, Lipofectin, LipofectAce, Cellfectin	Cationic lipid (proprietary)
		Calcium Phosphate Transfection System	$CaPO_4$
MBI Fermentas	www.fermentas.com	ExGen 500	Cationic polymer
Novagen	www.novagen.com		
Promega	www.promega.com	Transfast Transfection	Cationic lipid
		Tfx Reagents	Cationic lipid
		Transfectam	Cationic lipid
		ProFection	$CaPO_4$ or DEAE-Dextran
QIAGEN	www.qiagen.com	SuperFect	Activated dendrimer
		Effectene	Nonliposomal lipid and DNA condensing agent
		Transfection Reagent	Enhancer
		Selector Kit	Both reagents
Quantum Biotechnologies	www.quantumbiotech.com	GeneSHUTTLE 20 and 40	Cationic lipid
Sigma Aldrich	www.sigma-aldrich.com	DEAE Dextran Kit	DEAE-Dextran
		Calcium Phosphate Transfection	$CaPO_4$
		Escort, DOTAP, DOPE	Cationic lipid kits
Stratagene	www.stratagene.com	LipoTaxi	Liposome-mediated
		MBS Mammalian Transfection Kit	Modified $CaPO_4$
		Mammalian Transfection Kit	$CaPO_4$ and/or DEAE-Dextran
		Primary ENHANCER Reagent	Supplemented with lipid, $CaPO_4$
Wako Chemicals USA	www.wakousa.com	GeneTransfer HMG-1, -2 Mixture	Liposome-mediated

may be useless for another. Many of the protocols described in this chapter have been optimized for the standard lines of cultured cells. When using more exotic lines of cells, it is important to compare the efficiencies of several different methods. The protocols in this chapter present commonly used transfection techniques as well as methods that have proven successful with cell lines that are resistant to transfection by standard techniques. Commercial kits are available that provide collections of reagents for many types of transfections (please see Table 16-2).

Many techniques used in eukaryotic cell culture are not discussed in detail in this manual (for specific information on cell culture, please see Volume 1 of *Cells: A Laboratory Manual* [Spector et al. 1998a]). In particular, it is assumed that the conditions for optimal growth and passage of the cell lines to be used in this protocol have already been established.

The students study molecules now, spinning models across their computer screens and splicing the genes of one creature into those of another. The science of genetics is utterly changed... . Sometimes I wonder where we have misplaced our lives.

Andrea Barrett
"*The Behavior of the Hawkweeds.*"